Influence of Molecular Weight and Cristallinity of Poly(L-Lactic Acid) on the Adhesion and Proliferation of Human Osteoblast Like Cells

A.J. Salgado[1,2,a], Y. Wang[1,2,b], J.F. Mano[1,2,c*], R.L. Reis[1,2,d]

1- 3B's Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho, Campus de Gualtar, Braga

2- Department of Polymer Engineering, University of Minho, Campus de Azurém, Guimarães

[a]asalgado@dep.uminho.pt, [b]ywang@dep.uminho.pt, [c]jmano@dep.uminho.pt, [d]rgreis@dep.uminho.pt

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Abstract. The molecular weight and crystallinity of systems based on poly(L-lactic acid) PLLA is an important issue as it can influence, besides the general physical properties of the polymer, the patterns of cell adhesion, proliferation and cell morphology. The objective of the present study was to evaluate how crystallinity and molecular weight of PLLA influence the referred parameters. Four conditions were tested: low molecular weight amorphous and semi-crystalline PLLA disks, and high molecular weight amorphous and semi-crystalline PLLA disks, obtained from hot press. The thermal properties of the studied materials were accessed by differential scanning calorimetry. For the cell culture studies a human osteosarcoma cell line (SaOS-2) was chosen. Disks were immersed in a cell suspension containing 5×10^4 cells/ml and kept in culture for periods up to two weeks. Cell viability and proliferation of SaOS-2 cells was assessed by MTS test and a total protein assay, respectively. The adhesion and morphology of SaOS-2 cells on PLLA disks was assessed by scanning electronic microscopy. Results showed that cell viability was not affected by the different tested conditions. However, cell proliferation was increased in the high molecular weight amorphous samples and cells seemed to have higher adhesion patterns on semi-crystalline samples. This is probably happening due to different rates of integrin interaction with the substrate leading to different patterns of focal adhesion points formation.

Introduction

Poly(L-lactic acid), PLLA, is an aliphatic polyester that has been extensively used within the biomedical field, namely as pins, screws and plates for internal bone fixation, as well in systems for controlled drug delivery [1-3]. More recently this biodegradable polymer was put forward as a possible material for scaffolding in bone tissue engineering related applications [4,5]. As it is a biodegradable polymer approved by regulatory authorities for patient applications, it has been extensively studied. For instance it is known that in *in vivo* environments it degrades by non-specific hydrolysis, being its products lately incorporated in the different metabolic cycles present in the human body [6]. Its degradation is commonly characterized as being "bulk" with a sequential loss of molecular weight. However, and quite surprisingly, to our knowledge the influence of parameters such as crystallinity and molecular weight on the behaviour of human osteoblast like cells has not been studied. When looking to the literature only a handful of papers refer this subject [7-10], and even so they do not combine these two characteristics. Nevertheless they are extremely important. For instance the molecular weight is related with the degradation kinetics of the implant/scaffold. A low molecular weight leads to a faster degradation, which in turn can lead to a raise in the local pH, which then can cause cell death and therefore jeopardizing the integration of the implant on the affected area. On the other hand, a main feature of PLLA is its intrinsic low rate of crystallisation [11-13], being possible to produce polymer with different degrees of crystallinity

[14]. This parameter, and the general lamellar organisation, may be controlled by adequate thermal histories [15] and will have an influence on properties such as mechanical performance, as well as on its rate of hydrolytic or enzymatic degradability [15-19]. The crystallinity is also very important for phenomena such as cell adhesion. Crystalline surfaces have in general higher surface energies when compared to amorphous samples [7]. This differences in surface energy can then lead not only to different protein adsorption patterns, but at the same time, to different protein conformations, which could cause different adhesion patterns and morphological features by cell populations of osteogenic nature, leading in this sense to different cell proliferation rates. This is even more important because, as Park and Cima reported [7], slight alterations in the processing methodology can lead to important differences on the surface's characteristics. The objective of the present study was to evaluate how the molecular weight and levels of crystallinity of PLLA based systems influence the referred parameters.

Materials and Methods

The used materials were obtained from Purac Biochem with two inherent viscosities: 1.75 dl/g (PLLA1) and 5.87 dl/g (PLLA2). The molecular weight $\langle M_n \rangle$ and $\langle M_w \rangle$ of PLLA1 are 86 000 and 151 000, respectively, and for PLLA2, $\langle M_n \rangle$ and $\langle M_w \rangle$ are 269 000 and 301 000, respectively [20]. Amorphous structures were prepared by melting the materials in a hot plate (at ca. 200 ^{o}C), pressed between two metallic discs and quenched them in cold water. No crystalline reflection was detected in the WAXS spectra [20]. Semi-crystalline structures were prepared by the heating of the initially amorphous samples from 90 ^{o}C to 165 ^{o}C at a rate of 2 K/min, then hold for 30 min, and then quenched to room temperature in the air.

A Perkin-Elmer DSC 7 was used to evaluate the thermal behaviour of the materials. The experiments were performed from 30 ^{o}C to 200 ^{o}C at 10 K/min, using nitrogen as a purge gas. The temperature and heat transition of the instrument were calibrated with indium, also at 10 K/min. Samples' weight was about 7.5 mg.

For the cell culture studies an osteoblast like cell line, SaOS-2 (European Tissue Culture Collection - ETCC), was selected. This cell population has previously shown to express typical proteins of the osteogenic phenotype [21,22], such as osteopontin, and therefore can be considered as adequate for *in vitro* bone related studies. Cells were grown as monolayers using DMEM low glucose medium supplemented with 10% Foetal Bovine Serum (FBS, Biochrome, USA) and 1% antibiotics and antimycotics mixture (Gibco, USA) until they reached confluency. At this stage cells were trypsinized, centrifuged and ressuspended to a final density of $5x10^4$ cells/ml. Following this step, human osteoblast like cells were put in direct contact with the PLLA samples (1 ml cell suspension/sample), that had been previously placed in 24 well culture trays, and allowed to grow for 2 weeks. Cell viability was assessed by the MTS test while cell proliferation was screened by using a total protein assay. Both of these parameters were analyzed after 14 days (n=3/group) in culture. Finally the morphological features as well as cell adhesion to the different substrates were assessed by scanning electron microscopy (SEM), using a LEICA Cambridge S-360 (UK) apparatus. For this purpose cells were firstly fixed in a 2.5% glutaraldheyde, followed by a dehydration trhough an ethanol series and final air dried. Prior to analysis, samples were gold coated.

Results and Discussion

To better understand the thermal properties of the studied materials, both PLLA1 and PLLA2 were investigated using differential scanning calorimetry, DSC. Figure 1 shows the results obtained for both amorphous and semi-crystalline forms of the two polymers. For the amorphous materials an exothermic cold-crystallisation peak is observed after the glass transition. A more detailed analysis of cold-crystallisation process in PLLA was carried out elsewhere [20,23]. It is clear, and expectable, the absence of this process for the semi-crystalline materials, but the melting peak was found to be more complex, due to melting/recrystallisation processes. The endothermic enthalpy of the melting of crystallized PLLA1 is calculated from 158.0 to 182.6 ^{o}C, and for PLLA2 from 162.7

to 187.6 $^{\circ}$C. Compared with that of an infinitely large crystal, taken as 93.0 J/g [24], the obtained degrees of crystallinity are about 61% and 49% for PLLA1 and PLLA2, respectively.
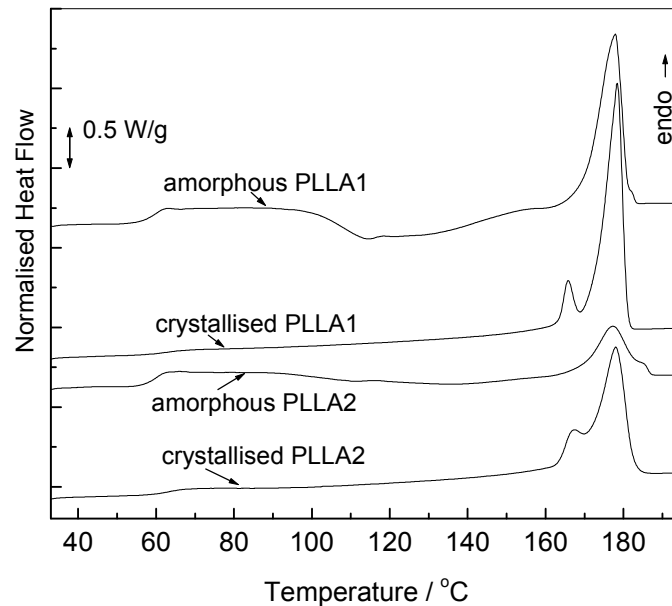

Figure 1 - DSC thermograms of the amorphous and semi-crystalline forms of PLLA1 and PLLA2 during heating at 10 K/min.
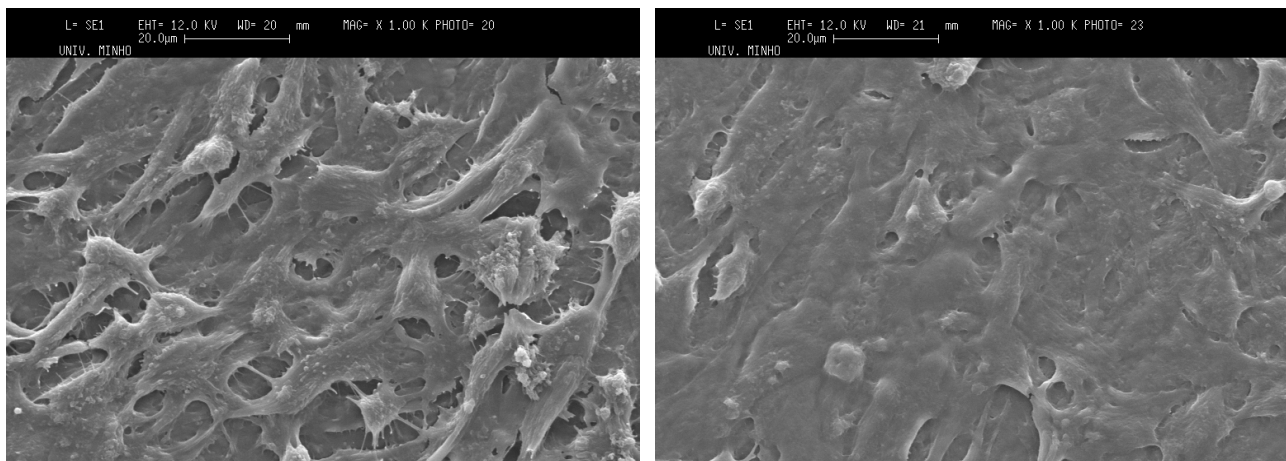

Figure 2 – SEM micrographs of human osteoblasts like cells after two weeks in culture (left-amorphous PLLA1; right- semi-crystalline PLLA1, scale bar: 20 μm).

The mechanism that dominates initial cell attachment to biomaterials' surface, *in vitro*, is the adsorption of proteins from cell culture medium serum on its surface [25]. This film of protein acts *in vitro*, as the matrix synthesized *in vivo* by bone forming cells and, its nature, extent and stability is to a large extent determined by the surface properties of the material and is thus a reflection of the substrate's characteristics [26,27]. Regarding the present experiment it was shown that after two weeks human osteoblast like cells were disclosing different morphologies when seeded on amorphous (A) or semi-crystalline (SC) substrates (Figure 2).

As it can be observed on the referred figures cells grown on the SC samples exhibit a more flattened and spread morphology, indicating a higher degree of adhesion strength, when compared to a more globule cellular form for the cells grown on the A samples. These differences in cell behaviour can be attributed to the different surface characteristics of the tested samples. It is known that the rate of protein adsorption is higher for materials that have higher surface energy values, and that crystalline materials disclose higher surface energy values when compared to non-crystalline [7]. Therefore we believe that the described phenomena may be affecting the morphological features of the human

osteoblast like cells cultured on PLLA disks, which are probably caused by a different rate of integrin expression and interaction with the substrate resulting in different formation patterns of focal adhesion points, as already been reported by others [28,29]. MTS results revealed that no major differences were found in terms of cell viability (Figure 3a), indicating that the low molecular weight samples, that could be degrading faster and should then decrease more the local pH, were not drastically reducing the viability of SaOS-2 cells. However, the same did not happen for the cell proliferation assays. As it can be observed in Figure 3b, amorphous PLLA2 samples disclosed higher levels of cell numbers when directly compared with the other tested samples. These results are similar to those obtained with 3T3 fibroblasts by Park and Cima [7]. The reasons why this phenomenon happens are not clear, and not clearly explained in the literature. For instance, in the above mentioned paper where 3T3 fibroblasts were used no explanation was put forward. A possible reason for the results obtained is that integrin interactions with the proteins adsorbed on the surface of the samples also have a strong role in this process. The proteins adsorbed on the surface of the different tested samples may, for the particular case of the SC samples, improve cell adhesion strength, whereas, for the A samples, enhance cell proliferation. This particular aspect should be further studied in future experiments.
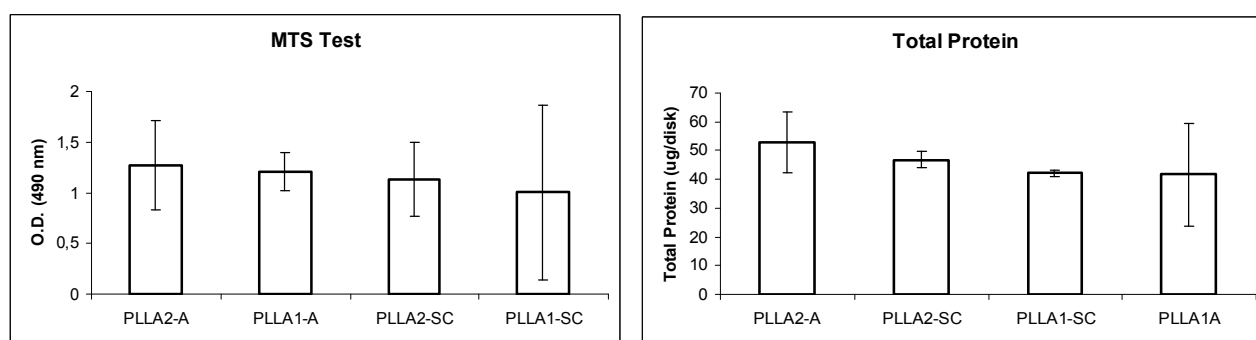

Figure 3 – a) Cell viability measured from the optical density (O.D.) at 490 nm and b) Cell proliferation assays on SaOS-2 cells grown on PLLA disks, (PLLA 1- low molecular weight; PLLA2- high molecular weight; A- amorphous; SC- Semi-crystalline).

Conclusions

The present work aimed to screen μ possible differences of human osteoblast cell behavior when cultured in PLLA substrates with different crystallinities and molecular weights. Results showed that molecular weight did no considerably affected cell behaviour. However, the latter was affected by the levels of crystallinity of the tested samples, namely regarding morphological features and cell proliferation. It is believed that the rate of protein adsorption on the different samples and consequent integrin interaction and focal adhesion point formation with it may have a role on this phenomenon.

Acknowledgements

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